IN THE SPECIFICATION[1]

Please replace the paragraph on page 1, line 10 with the following paragraph:

This application is a continuation of United States Application No. 08/031,801 filed March 15, 1993, which is a continuation-in-part of United States Application No. 07/919,297, filed July 24, 1992, which is a continuation-in-part of United States Application No. 07/610,515, filed November 8, 1990, which is a continuation-in-part of United States Application No. 07/466,008, filed January 12, 1990, the entire disclosures of which are all incorporated herein by reference.

Please delete the paragraph on page 7, lines 23-24, and replace it with the following paragraph:

Figure 6 (SEQ ID NOS 5-6, 8-9, 27-28, 29-30) is a diagram of the derivation of the plasmid pK.TK/Neo, as described in Example III, infra.

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[1] An "Appendix of Amendments" is enclosed herewith showing the amendments to the specification. In the Appendix, additions are underscored and deletions are bracketed.

Please delete the paragraph on page 7, lines 35-37, and replace it with the following paragraph:

Figure 9 (SEQ ID NOS 5-6, 29-30, 8, 31, 10-11) is a diagram of the construction of vectors for inactivating the kappa light chain J and constant regions as described in Example IV, infra.

Please delete the paragraph on page 31, lines 22-37, thru page 32, lines 1-14 and replace it with the following paragraph:

ES colonies resulting 10-14 days after electroporation are picked with drawn out capillary pipettes for analysis using PCR. Half of each picked colony is saved in 24-well plates already seeded with mitomycin-treated feeder cells. The other halves, combined in pools of 3-4, are transferred to Eppendorf tubes containing approximately 0.5 ml of PBS and analyzed for homologous recombination by PCR. Conditions for PCR reactions are essentially as described (Kim and Smithies (1998), Nucleic Acids Res. 16:8887-8893). After pelleting, the ES cells are resuspended in 5 $\mu$l of PBS and are lysed by the addition of 55 $\mu$l of $H_2O$ to each tube. DNAses are inactivated by heating each tube at 95°C for 10 min. After treatment with proteinase K at 55°C for 30 min, 30 $\mu$l of each lysate is transferred to a tube containing 20 $\mu$l of a reaction mixture including PCR buffer: 1.5 $\mu$g of each primer, 3U of Taq polymerase, 10% DMSO, and dNTPs, each at 0.2 mM. The PCR expansion employs 55 cycles using thermocycler with 65 seconds melt at 92°C and a 10 min annealing and extension time at 65°C. The two priming oligonucleotides are TGGCGGACCGCTATCCCCCAGGAC (SEQ ID NO: 1) and

TAGCCTGGGTCCCTCCTTAC (SEQ ID NO: 2), which correspond respectively to a region 650 bases 3' of the start codon of the neomycin gene and sequences located in the mouse heavy chain gene, 1100 bases 3' of the insertion site. 20 μl of the reaction mix is electrophoresed on agarose gels and transferred to nylon membranes (Zeta Bind). Filters are probed with a $^{32}$P-labelled fragment of the 991 bp XbaI fragment of the J-C region.

Please delete the paragraph on page 33, lines 5-37 thru page 34, lines 1-16, and replace it with the following paragraph:

The ES cell line E14TG2a (Koller and Smithies (1989), PNAS USA, 86:8932-8935) was cultured on mitomycin C-treated embryonic fibroblast feeder layers as described (Koller and Smithies (1989), PNAS USA, 86:8932-8935). ES cells were trypsinized, resuspended in HBS buffer (pH7.05; 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.7 mM $Na_2HPO_4$, 21 mM HEPES pH 7.1) at a concentration of $2X10^7$/ml and electroporated in the presence of 50 μg/ml of the linearized inactivation vector. Electroporation was carried out with a BioRad Gene Pulser using 240 volts and 500μF capacitance. $5X10^6$ electroporated cells were plated onto mitomycin C-treated fibroblasts in 100 mm dishes in the presence of Dulvecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum and 0.1 mM 2-mercaptoethanol. The media was replaced 24 hr after electroporation with media containing 200 μg/ml G418. G418-resistant ES colonies resulting from growth 12-14 days after electroporation were picked with drawn out capillary pipettes for analysis using the polymerase chain reaction (PCR). Half of each picked colony was transferred to an individual well

of a 24-well plate, already seeded with mitomycin C-treated feeder cells. The other halves, combined in pools of four, were transferred to Eppendorf tubes containing 0.3 ml of PBS and cell lysates were prepared for PCR analysis as described by Joyner et al (1989) Nature, 338:153-155. The PCR reaction included 5-20 $\mu$l of the cell lysate, 1 $\mu$M of each primer, 1.5 U of Taq polymerase and 200 $\mu$M of dNTPs. The PCR amplification employed 45 cycles using a thermal cycler (Perkin-Elmer Cetus), with 1 min. melt at 94°C, 2 min. annealing at 55°C, and 3 min. extension at 72°C. The two priming oligonucleotides are ACGGTATCGCCGCTCCCGAT (SEQ ID NO: 3) and AGTCACTGTAAAGACTTCGGGTA (SEQ ID NO: 4), which correspond respectively to about 120 bases 5' of the BamHI site of the neomycin gene, and to the sequences located in the mouse heavy chain gene, about 160 bases 3' of the insertion site. Successful homologous recombination gives rise to an about 1.4 kb fragment. 20 $\mu$l of the reaction mixture is electrophoresed on 1% agarose gels, stained with ethidium bromide and transferred to nylon membranes (Gene Screen). Filters were probed with a $^{32}$P-labelled EcoRI-PstI about 1.4 kb fragment located in the mouse heavy chain, 3' of the insertion site (see Figure 2). For further analysis, genomic DNA was prepared from ES cells, digested with restriction enzymes as recommended by the manufacturers, and fragments were separated on 1% agarose gels. DNA was transferred to nylon membranes (Gene Screen) and probed with the $^{32}$P-labelled fragment as described above.

Please delete the paragraph on page 41, lines 31-37, thru page 42, line 1, and replace it with the following paragraph:

A 4.0 kb SphI/Bsu361 fragment from the plasmid pUC218/5.6kappa was subcloned into the SphI and Bsu361 sites of the vector pSK.A to give the plasmid pSK.A/5'K. The vector pSK.A is a modification of pBluescript SK-which has a synthetic polylinker:

(SEQ ID NOS 5 & 6, respectively, in order of appearance)

5' GCATATGCCTGAGGTAAGCATGCGGTACCGAATTCTATAAGCTTGCGGCCGCAGCT

CATGCGTATACGGACTCCATTCGTACGCCATGGCTTAAGATATTCGAACGCCGGCG 3'

inserted between the pBluescript KpnI and SacI sites.

Please delete the paragraph on page 42, lines 2-9, and replace it with the following paragraph:

A 2.7 kb EcoRI/HindIII fragment containing the herpes thymidine kinase (TK) gene driven by the mouse phosphoglycerate kinase gene (PGK) promoter from the plasmid pKJtk (Tybulewicz, et al. (1991) Cell 65:1153-1163) was inserted into the EcoRI and NotI sites of pSK.A/5'K by using a HindIII/NotI adapter with the sequence:

5' AGCTGGAACCCCTTGCCCTTGGGGAACGCCGG 3' (SEQ ID NO: 7).

Please delete the paragraph on page 42, lines 14-22, and replace it with the following paragraph:

A 1.1 kb XhoI/BamHI fragment from pMC1Neo, which contains the mammalian drug selectable marker for resistance to neomycin, was cloned into the XhoI and BamHI sites of the plasmid pSK.B to give the plasmid pSK.B/Neo. The vector pSK.B is a modification of pBluescript SK-which has a synthetic polylinker:

(SEQ ID NOS 8-9, respectively, in order of appearance)

5' GAGCTCGGATCCTATCTCGAGGAATTCTATAAGCTTCATATGTAGCT

CATGCTCGAGCCTAGGATAGAGCTCCTTAAGATATTCGAAGTATACA 3'

inserted between the pBluescript KpnI and SacI sites.

Please delete the paragraph on page 42, lines 25-37, and replace it with the following paragraph:

A 1.1 kb BglII/BamHI fragment from pUC218/5.6kappa, which contains homology to the 3' end of the kappa region, was cloned into BamHI digested, alkaline phosphatase treated pSK.C vector. The vector pSK.C is a modification of pBluescript SK- which has a synthetic polylinker:

(SEQ ID NOS 10-11, respectively in order of appearance)

5' AAGCTTATAGAATTCGGTACCTGGATCCTGAGCTCATAGCGGCCGCAGCT

CATGTTCGAATATCTTAAGCCATGGACCTAGGACTCGAGTATCGCCGGCG 3'

inserted between the pBluescript KpnI and SacI sites. The resulting plasmid, pSK.C/3'K is oriented such that transcription proceeds from the SacI site in the plasmid polylinker in the direction of the KpnI site.

Please delete the paragraph on page 48, lines 19-31, and replace it with the following paragraph:

The polylinker of the plasmid pBluescript SK was modified by cloning between the KpnI and SacI sites a synthetic polylinker defined by the oligonucleotides 5'-GCATATGCCTGAGGGTAAGCATGCGGTACCGAATTCTATAAGCTTGCGGCCGCAGCT-3' (SEQ ID NO: 5) AND 5'-

GCGGCCGCAAGCTTATAGAATTCGGTACCGCATGCTTACCTCAGGCATATGCGTAC-3' (SEQ ID NO: 6) to create the plasmid pSK.A, 5'GAGCTCGGATCCTATCTCGAGGAATTCTATAAGCTTCATATGTAGCT-3' (SEQ ID NO: 8) and 5'-ACATATGAAGCTTATAGAATTCCTCGAGATAGGATCCHAGCTCGTAC-3' (SEQ ID NO: 12) to create plasmid pSK.8, 5'-AAGCTTATAGAATTCGGTACC TGGATCCTGAGCTCATAGCGGCCGCAGCT-3' (SEQ ID NO: 10) to create plasmid psK.B and 5'-GCGGCCGCTATGAGCTCAGGATCCAGGTACCGAATTCTATAAGCTTG TAC-3' (SEQ ID NO: 11) to create the plasmid pSK.C.

Please delete the paragraph on page 48 line 32-37, thru page 49, lines 1-33, and replace it with the following paragraph:

A diphtheria toxin gene cassette was created in which the gene was flanked by the PGK promoter and the bovine growth hormone polyadenylation signal (Woychik et al. (1984), Proc. Natl. Acad. Sci. U.S.A, 81: 3944-3948; Pfarr et al. (1986), DNA 5:115-122). A 2.3 kb XbaI/EcoRI fragment from pTH-1 (Maxwell et al. (1986), Cancer Res. 46:4660-4664) containing the diphtheria toxin A chain driven by the human metallothionein (hMTII) promoter was cloned into pBluescript SK cut with XbaI and EcoRI to give the plasmid pSK.DT. The hMTII promoter of pSK.DT was replaced with the PGK promoter from pKJ1 (Tybulewicz et al. (1991), Cell 65:1153-1163). A 0.5 kb XbaI/PstI fragment from pKJ1 was joined to a 3.1 kb XbaI/NcoI fragment from pSK.DT using a PstI/NcoI adapter formed from the oligonucleotides 5'-GGGAAGCCGCCGC-3' (SEQ ID NO: 13) and 5'-CATGGCGGCGGCTTCCCTGCA-3' (SEQ ID NO: 14 ) to give the plasmid pSK.pgkDT. A 248 bp fragment containing the bovine growth hormone polyadenylation signal, obtained by PCR amplification

of bovine genomic DNA using the oligonucleotide primers 5'-CAGGATCCAGCTGTGCCTTCTAGTTG-3' (SEQ ID NO: 15) and 5'-CTGAGCTCTAGACCCATAGAGCCCACCGCA-3' (SEQ ID NO: 16), was cloned into pCR1000 (Invitron Corp., San Diego, CA). The polyadenylation sequence was then cloned behind the DT gene as a HindIII/PvuII fragment into pSK.pgkDT cut with HindIII and HpaI to give the plasmid pSK.pgkDTbovGH. The DT gene cassette from pSK.pgkDTbovGH was moved as a 2.1 kb EcoRI/HindIII fragment into pSK.A cut with EcoRI and NotI using a HindIII/NotI adapter formed from the oligonucleotides 5'-AGCTGGAACCCCTTGC-3' (SEQ ID NO: 17) and 5'-GGCCGCAAGGGGTTCC-3' (SEQ ID NO: 18) to give the plasmid pSK.A/DT. Between the SphI and Bsu36I sites of both pSK.A and pSK.A/DT the 5' region of homology for the kappa locus was cloned. For this purpose a 4.0 kb SphI/Bsu36I fragment resulting from a partial Bsu36I digest followed by a complete SphI digest of plasmid subclone pUC218/5.6kappa was ligated to pSK.A or pSK.A/DT to give the plasmids pSK.A/5'K and pSK.A/DT/5'K, respectively. In the plasmid, pSK.A/DT/5'K, the 5'-end of the DT gene and kappa fragment were adjacent to each other running in the opposite transcriptional orientations.

Please delete the paragraph on page 56, lines 7-33, and replace it with the following paragraph:

PCR primers for the human VH6 gene (V6A= 5' GCA GAG CCT GCT GAA TTC TGG CTG 3' (SEQ ID NO: 19) and V6B= 5' GTA ATA CAC AGC CGT GTC CTG G 3' (SEQ ID NO: 20)) were used to screen DNA pools from the Washington University human YAC library (Washington University, St. Louis, MO). Positive pools were

subsequently screened by colony hybridization and one positive microtiter plate well, A287-C10, was identified. Two different sized (205 kb and 215 kb) VH6-containing YACs were isolated from the mocrotiter well. In addition to VH6, the smaller of the two IgH YACs, A287-C10 (205 kb), hybridized to probes for the following sequences: delta, mu, JH, D, VH1, VH2, and VH4. The larger of the two IgH YACs, A287-C10 (215 kb), hybridized to the following probes: delta, JH, D, VH1, VH2 and VH4, but not to mu. The YACs contained sequences from at least 5 VH genes including two VH1 genes, one VH2, one VH4 and one VH6 gene. Analysis of restriction digests indicated that the 205 kb YAC contains a deletion (about 20 kb size) that removes some, but not all of the D gene cluster, with the remainder of the YAC appearing to be intact and in germline configuration. PCR and detailed restriction digest analysis of the 205 kb YAC demonstrated the presence of several different D gene family members. The 215 kb YAC appeared to contain the complete major D gene cluster but had a deletion (about 10 kb) that removed the mu gene. This deletion does not appear to affect the JH cluster or the enhancer located between JH and mu genes.

Please delete the paragraph on page 57, lines 34-37, thru page 60, lines 1-3, and replace it with the following paragraph:

Southern analysis of the 230 kb A287-C10 YAC targeted with pLUTO was carried out using a variety of probes to demonstrate the intact, unrearranged nature of the cloned, human IgH sequences. In most cases, the results of BamHI, HindIII and EcoRI digests were compared to restriction data

for WI38 (a human embryonic fetal lung-derived cell line), the 205 kb and 215 kb deletion-derivatives of A287-C10 and to published values. The diversity (D) gene profile determined by hybridization with a D region probe (0.45 NcoI/PstI fragment; Berman et al., 1988) demonstrated the expected four D gene segments (D1-D4 (Siebenlist et al., 1981; Nature 294; 631-635). For example, with BamHI, four restriction fragments, 3.8 kb, 4.5 kb, 6.9 kb and 7.8 kb, were observed in A287-C10 and WI38. WI38 had one additional larger band, presumed to originate from the chromosome 16 D5 region (Matsuda et al., 1988, EMBO 7:1047-1051). PCR and Southern analysis with D family-specific primers and probes demonstrated in the 215 kb deletion-derivative YAC (which appeared to have an intact D region with the same restriction pattern as the 230 kb YAC) the presence of 2 to 4 members of each of the following D gene families: DM, DN, DK, DA, DXP and DLR. The J-mu intronic enhancer, which was sequenced from cloned PCR products from the A287-C10 230 kb YAC (primers EnA = 5'TTC CGG CCC CGA TGC GGG ACT GC 3' (SEQ ID NO: 21) and EnB1 = 5'CCT CTC CCT AAG ACT 3' (SEQ ID NO: 22) and determined to be intact, also generated single restriction fragments of approximately the predicted sizes with BamHI, ExoRI and HindIII when probed with the 480 bp PCR product. The JH region was evaluated with an approximately 6 kb BamHI/HindIII fragment probe spanning DHQ52 and the entire JH region (Ravetch et al., 1981, Cell 27: 583-591). A287-C10 generated restriction fragments of approximately the expected sizes. Furthermore, the same-sized restriction fragments were detected with the enhancer and the JH probes (Ravetch et al., supra; Shin et al., 1991, supra). The approximately 18 kb BamHI JH fragment detected in A287-C10 and WI38 also

hybridized to a 0.9 kb mu probe sequence (Ravetch et al., supra). Hybridization with the 0.9 kb EcoRI fragment mu probe (Ravetch et al., supra) showed restriction fragments of approximately the expected sizes (Ravetch et al., supra; Shin et al., supra): > 12 kb BamHI (approximately 17 kb expected); 0.9 kb EcoRI (0.9 kb expected) and approximately 12 kb HindIII (approximately 11 kb expected). WI38 gave the same-sized BamHI fragment as A287-C10. The JH and DHQ52 regions were sequenced from both of the deletion derivative YACs and both were in germline configuration. Delta was analyzed with an exon 1 PCR product (containing the approximately 160 bp region between primers D1B= 5′ CAA AGG ATA ACA GCC CTG 3′ (SEQ ID NO: 23) and D1D = 5′ ACG TGG CTG CTT GTC ATG 3′ (SEQ ID NO: 24)); restriction fragments for A287-C10 were close to those expected from the literature (Shin et al., supra) and to those determined for WI38. The 3′ cloning site of the YAC may be the first EcoRI site 3′ of delta (Shin et al., supra) or another EcoRI site further 3′. VH gene probes for VH1, VH4 and VH6 (Berman et al., supra), and for VH2 (Takahashi et al., 1984, Proc. Nat. Acad. Sci. USA 81: 5194-5198) were used to evaluate the variable gene content of the YAC. A287-C10 contains two VH1 genes that approximate the predicted sizes (Shin et al., supra; Matsuda et al., 1993, supra); restriction analysis with the three enzymes gave close to the expected fragment sizes; e.g. with EcoRI observed bands are 3.4 and 7.8 kb (expected are 3.4 and 7.2 kb). The predicted size EcoRI fragments for VH4 (5.3 kb observed, 5.1 kb expected) and for VH6 (0.8 kb observed, 0.9 kb expected) (Shin, et al., supra; Matsuda et al., supra) were present in A287-C10. The expected size EcoRI fragment was seen for VH2 (5.5 kb observed, 5.4 kb expected), but the BamHI and HindIII

fragments were different from those predicted. Coincident hybridization of the BamHI and HindIII fragments with a pBR322 probe suggested that the EcoRI site which is at the 5' end of the VH2 gene (Shin et al., supra) is the 5' cloning site, thus eliminating the natural 5' HindIII site and BamHI sites. The overall size of the YAC insert (estimated to be approximately 220 kb) fits well with the predicted size for an intact, unrearranged segment starting at the 5' end of the 3'-most VH2 gene and extending to an EcoRI site 3' of the delta locus (Shin et al., supra).

Please delete the paragraph on page 66, lines 35-37, thru page 67, lines 1-19, and replace it with the following paragraph:

The abiliy of ESY cells to repopulate mice, including the germline, was demonstrated by microinjection of ES cells into mouse blastocysts and the generation of chimeric mice. ESY cells were microinjected into C57BL/6J mouse blastocysts, and chimeric mice were generated as previously described. Chimeric males were mated with C57BL/6J females and germline transmission was determined by the presence of agouti offspring. Genomic DNA prepared from the tails of the chimeric mice were analyzed for the presence of the yHPRT DNA in the mouse genome by PCR analysis. The presence of the YAC left arm was analyzed using the two priming oligonucleotides, 5' TTCTCGGAGCACTGTC CGACC (SEQ ID NO: 32) and 5' CTTGCGCCTTAAACCAACTTGGTACCG (SEQ ID NO: 33), which were derived, respectively, from the pBR322 sequences and the SUP4 gene within the YAC left vector arm. A 259 bp PCR product was obtained from the analysis of the yeast containing yHPRT and the ESY cell lines. PCR analysis of tail DNA prepared

from 18 chimeric mice generated from ESY cell lines ESY3-1, ESY3-6 and ESY5-2, gave rise to the expected PCR product, thus indicating the presence of the YAC left vector arm in the genome of the chimeric mice.

Please delete the paragraph on page 68, lines 1-35, and replace it with the following paragraph:

Using a human HPRT-specific PCR assay on mRNA-derived cDNAs from a yHPRT-containing offspring, the expression of the human HPRT gene in all the tissues tested was detected (Figure 15 A and B), thus demonstrating the transmitted YAC retained its function with fidelity. In this experiment, human HPRT mRNA was detected by reverse transcription (RT)-PCR in ES, ESY 3-1 and Hut 78 (human) cells, spleen and liver from a control mouse (C) or the 4-3 agouti offspring (derived from the 394/95-2 chimera) and a sample containing no template DNA (indicated as "_" in Figure 15A). Reverse transcription of poly (A+) RNA and PCR amplification of specific cDNA sequences were performed using the cDNA Cycle Kit (Invitrogen). Specific amplification of a 626 bp fragment from human HPRT or cDNA in the presence of murine HPRT cDNA was performed as outlined by Huxley et al., supra. Integrity of all RNA samples was demonstrated by PCR amplification of cDNAs for the mouse □-interferon receptor. The primers used to amplify a 359 bp fragment were: GTATGTGGAGCATAACCGGAG (SEQ ID NO: 25) and CAGGTTTTGTCTCTAACGTGG (SEQ ID NO: 26). The human HPRT and the □-interferon receptor primers were designed to eliminate the possibility of obtaining PCR products from genomic DNA contamination. PCR products were analyzed by electrophoresis and visualized with ethidium bromide. The size markers are 1